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A-TYPE PROANTHOCYANIDINS FROM STEM-BARK OF *PAVETTA OWARIENSIS*

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Key Word Index—*Pavetta owariensis*; Rubiaceae; stem-bark; flavan-3-ols; proanthocyanidins A-type; dimers.

Abstract—In a chemical investigation on the stem-bark of *Pavetta owariensis*, a new proanthocyanidin A-type, *ent*-epicatechin (4α→8,2α→*O*→7)-*ent*-catechin designated as pavetannin A1, was isolated as second representative of a (2α,4α) A-type proanthocyanidin. Additionally, the occurrence of (+)-catechin, (−)-epicatechin, *ent*-epicatechin, proanthocyanidin A-2 and proanthocyanidin A-4 was demonstrated, the latter being reported from a natural source for the second time.

INTRODUCTION

We previously described the antibacterial and anti-herpetic activities of extracts of *Pavetta owariensis* P. Beauv. when tested *in vitro*. Proanthocyanidins possessing doubly linked structures were shown to be the main active components [1]. As the synergistic action of proanthocyanidins on the anthelmintic activity of known anthelmintics has been demonstrated [2], this can provide some biological support for the use of *P. owariensis* as an anthelmintic [3]. During our investigations on the active components of *P. owariensis*, we have isolated three monomeric flavan-3-ols and three proanthocyanidin A-type compounds. In contrast to the ubiquitous B-type proanthocyanidins much less complete chemical data are available on doubly linked proanthocyanidins. We now report on the characterization of A-type proanthocyanidins from *P. owariensis*, based on spectroscopic techniques (UV, FABMS, ^{1}H and ^{13}C NMR), chemical transformation, and correlation with known substances. To the best of our knowledge, this is the first report on the occurrence of doubly linked proanthocyanidins in the Rubiaceae.

RESULTS AND DISCUSSION

The acetone soluble portion of the ethanol 80% extract was fractionated by a combination of droplet counter current chromatography (DCCC) and Sephadex LH-20 chromatography to yield 0.001% (+)-catechin (**1**), 0.015% (−)-epicatechin (**2**), 0.010% (+)-epicatechin (**3**) and compounds **4**–**6**. The structures of **1** and **2** were established by chromatographic and spectroscopic comparisons with authentic samples.

Chromatographically, **3** was found to be different from **1** and **2**: TLC analysis on cellulose (Merck) with water [3] showed R_f values of 0.30, 0.27 and 0.24 for compounds **1**–**3**, respectively. The FAB mass spectrum of **3** and the EI mass spectrum of its peracetylated derivative (**3a**) were consistent with a monoflavanoid constitution. ^{1}H and ^{13}C NMR spectra of the free phenol **3** and its peracetate derivative **3a** were almost similar to those of (−)-epicatechin (**2**) and **2a**, respectively. The optical rotation of **3a**, however, showed a positive sign [+40.8°; Me_2CO, *c* 1.0] indicating that compound **3** is an enantiomer of (−)-epicatechin. Thus, **3** was identified as (+)-epicatechin or *ent*-epicatechin, which has already been found in various species of Palmae, *Polygonum multiflorum* (Polygonaceae) and *Uncaria gambir* (Rubiaceae) [4–6]. *ent*-Catechin and *ent*-epicatechin usually co-occurred with (−)-epicatechin and (+)-catechin. Ellis *et al.* [7] have proposed that the 2*S* units with a 2,3-*cis* configuration arise from 2*R* units with a 2,3-*trans* configuration by the action of an epimerase enzyme complex.

Compound **4** was isolated as a pale brown amorphous powder in a yield of 0.072% and responded positively to the vanillin–sulphuric or anisaldehyde–sulphuric acid reagents. Its UV spectrum showed a band at 279 nm corresponding to the two phenolic chromophores of the phloroglucinol and catechol rings. The positive FAB mass spectrum of **4** showed a $[M+H]^+$ ion at *m/z* 577, corresponding to a biflavanoid structure and consistent with a proanthocyanidin-A-type nature [8]. Fragmentation, characteristic for the interflavanoid bonds, was apparent from the fragment ion at *m/z* 287. Fragment ions formed by a retro-Diels–Alder fission and subsequent loss of water were observed at *m/z* 425 and 407. The ^{1}H and ^{13}C NMR spectral data of **4** (Tables 1 and 2) were superimposable with those of authentic proanthocyanidin A-2 (ProA2). The ^{1}H NMR spectrum of the nona-acetate derivative (**4a**) was also similar to that described for the nona-acetate of ProA-2 [9]. The ^{1}H and ^{13}C NMR spectra of **4** [in CD_3OD or $(CD_3)_2CO$] and **4a** (in $CDCl_3$), were characterized by the conspicuous absence of the effects of dynamic rotational isomerism at ambient temperatures when compared to those of B-type proanthocyanidins. This is due to the introduction of an additional bond between C-2 of the upper unit and the

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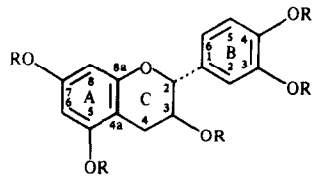

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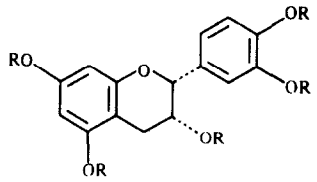
1a R = Ac



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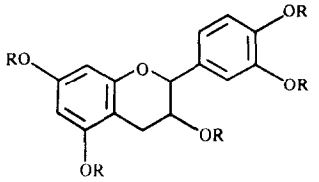

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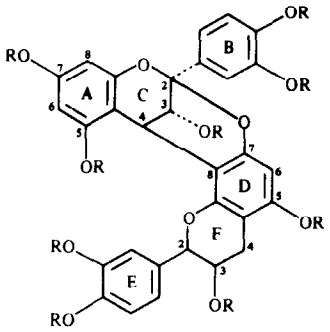

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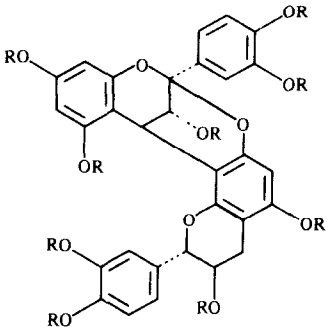

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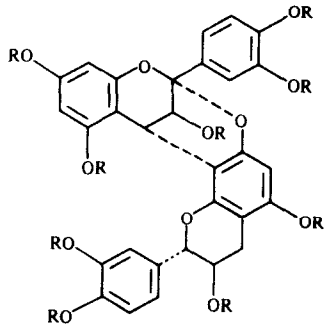

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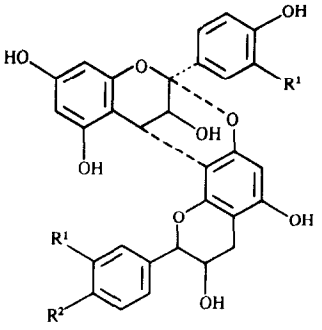
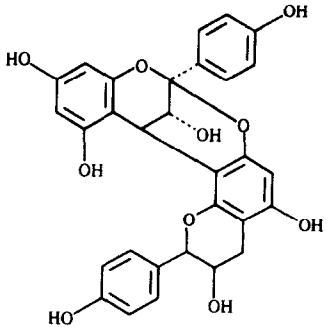
6a R = Ac



7 R^1 = OH, R^2 = OH
8 R^1 = H, R^2 = OH



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oxygen attached to C-7 of the bottom unit which produces a rigid six-membered ring [10]. Accordingly, compound **4** was identified as proanthocyanidin A-2 or epicatechin-(4β→8,2β→*O*→7)-epicatechin.

Compound **5**, obtained in a yield of 0.02%, showed a $[M+H]^+$ ion at *m/z* 577 in its FAB mass spectrum, suggesting an A-type proanthocyanidin dimer. This was further substantiated by the fragmentation pattern consistent with that of **4**. The ^1H and ^{13}C NMR spectra of **5** and its nona-acetate **5a** were similar to those of **4** and **4a**, respectively, the significant differences being only the chemical shifts of the heterocyclic protons and carbons

Table 1. ^{1}H NMR data for proanthocyanidins **1**–**6** (in methanol-d_4 and **5**–**6** also recorded in acetone-d_6) and their acetate derivatives **1a**–**6a** [in $CDCl_3$, δ-values; J-values (Hz) are given in parentheses]

	C-ring			F-ring		
Compound	**2**	**3**	**4**	**2**	**3**	**4**
1	4.47 (7.3)	3.88 *m*	2.41 *dd* (16.1, 8.3) 2.76 *dd* (16.1, 5.4)	—	—	—
1a	5.24 *m*	5.13 *m*	2.65 *dd* (16, 6.3) 2.86 *dd* (16, 5.8)	—	—	—
2	—	4.09 *br s*	2.68 *dd* (16, 4.2) 2.79 *dd* (16, 2.9)	—	—	—
2a	5.37 *m*	5.10 *br s*	2.89 *m*	—	—	—
3	—	4.01 *m*	2.66–2.83 *m*	—	—	—
3a	5.40 *m*	5.12 *br s*	2.88 *dd* (16, 6.7) 2.99 *dd* (16, 4.8)	—	—	—
4	—	4.44 *d* (4)	4.09 *d* (3)	—	4.28 *br s*	2.86 *m*
4a	—	5.21 *d* (4)	4.61 *d* (4)	5.23 *m*	5.23 *m*	2.88 *m*
5 (methanol-d_4)	—	4.13 *d* (4)	4.23 *m*	—	4.23 *m*	2.85 *m*
5 (acetone-d_6)	—	4.10 *d* (4)	4.27 *d* (4)	4.67 *d* (8)	4.00 *m*	—
5a	—	5.36 *d* (4)	4.75 *d* (4)	5.27 *m*	5.27 *m*	2.86 *m*
6 (methanol-d_4)	—	4.01 *d* (4)	4.36 *d* (3)	—	4.19 *br, s*	2.75 *dd* (8, 16) 2.88 *dd* (6, 16)
6 (acetone-d_6)	—	4.14 *br s*	4.34 *d* (4)	4.97 *br s*	4.24 *m*	—
6a	—	5.21 *m*	4.60 *d* (4)	5.27 *m*	5.18 *m*	2.86 *m*

—Overlapped with the solvent signals.

(Tables 1 and 2). In the ^{13}C NMR spectrum, the presence of signals at δ83.95 and 68.52 attributable to C-2 and C-3 of ring F, respectively, corresponded to a catechin terminal unit. Independent support for the presence of a 2,3-*trans* configurated 'lower' unit was available from the coupling constant (J = 8 Hz) of H-2 and H-3 (F). Furthermore, the unsubstituted A-ring carbon at 98.40 (H-6) was in agreement with a C-4/C-8 interflavanoid linkage [11]. The spectral data (^{1}H, ^{13}C NMR) of **5** were in close agreement with the data reported for proanthocyanidin A-4 [12] and a synthetic specimen [13]. Therefore, **5** was identified as epicatechin-(4β→8,2β→*O*→7)-*ent*-catechin or ProA-4.

Compound **6** was isolated as a pale brown amorphous powder in a yield of 0.042% and gave rise to orange and red colour reactions with anisaldehyde–sulphuric and vanillin–sulphuric acid reagents, respectively. On TLC (solvent B), compound **6** showed a R_f value of 0.49, which was different from those of **4** and **5** (R_f 0.57, 0.51, respectively). The FAB-mass spectrum indicated a $[M+H]^+$ ion at m/z 577 and a fragmentation pattern characteristic of a proanthocyanidin A-type dimer [8]. Comparison of ^{1}H NMR data of **5a** and **6a** (Table 1) revealed their close structural resemblance. Both displayed in the heterocyclic region AB systems (δ5.36, 4.75, both *d*, J = 4 Hz for **5a**; δ5.21 (*m*) and 4.60, *d*, J = 4 Hz for **6a**) characteristic of the C-ring protons of A-type proanthocyanidins. Similarly inspections of the heterocyclic and aromatic proton systems of the unsubstituted parent compounds **5** and **6** reflected their close structural likeness. The aromatic region of the spectra displayed AB systems (δ6.13, 6.08, both *d*, J = 2 Hz for **5**; δ5.96, 6.03, J = 2 Hz for **6**) for their respective A-rings. Notable differences between the spectra in methanol-d_4 included conspicuous shielding ($\Delta\delta$-0.12 ppm) of H-3 (C) and deshielding ($\Delta\delta$0.13 ppm) of H-4 (C) in **6** relative to **5**. A diagnostic feature of the ^{1}H NMR spectrum of **6** in acetone-d_6 was the downfield position H-2 (F) (δ4.97) relative to that of catechin (δ4.57) which implies steric interaction between H-2 (F) and the upper flavan aromatic ring [12], hence, suggesting a C-4/C-8 interflavanoid linkage. Since H-8 always resonates at lower field than H-6 [14, 15], the aromatic proton signals of **6** (in CD_3OD) at δ6.04 (singlet), 6.03 (*d*, J = 2 Hz), and 5.96 (*d*, J = 2 Hz) were attributed to H-6 (D), H-8 and H-6 (A), respectively. Acetylation with acetic anhydride–pyridine of **6** gave an amorphous nona-acetate **6a** which differed from **5a** by a slight shielding ($\Delta\delta$ − 0.15 ppm) of H-4 (C), a slight deshielding ($\Delta\delta$0.15) of H-3 (C) in **6a**. The aromatic A- and D-ring protons were observed downfield at δ6.82 (J = 2 Hz) for H-8 (A), δ6.49 and 6.50 [H-6 (A and D)]. This attribution is also consistent with a C-4/C-8 linked proanthocyanidin. As ProA-4 nona-acetate **5a**, compound **6a** showed an unusual high field signal at δ1.50 corresponding to a phenolic acetoxy function. This phenomenon may be rationalized by the shielding influence of the lower catechol E-ring on the oxygen substituent at C-5 compatible with the anticipated C-4/C-8 interflavanoid linkage [12]. Evidence supporting the stereochemical and interflavan linkage assignments was also available from the Spin-Echo Fourier Transform (SEFT) ^{13}C NMR spectra of **6** and its nona-acetate **6a**. The doubly linked structure of **6** was supported by the typical ketal carbon resonance at δ 104.3 due to the flavan C-2 (C) carbon. The position of the signals C-2 (F) at δ81.8 and C-3 (C) at δ68.1 clearly established that catechin was the terminal unit [11, 16, 17]. Since these signals were very similar to those of ProA-4, the terminal unit was found to probably be *ent*-catechin or (−)-catechin [12]. This was supported by the identical chemical shift of the C-2 (F)

Table 2. ^{13}C NMR data for compounds **1–6** (in CD_3OD) and their acetate derivatives **1a–6a** (in $CDCl_3$, δ-values)

Compound		C-Ring carbon (u) F-Ring carbon (l) 2	3	4	4a	A-Ring carbon (u) D-Ring carbon (l) 5[a]	6	7[a]	8	8a	B-Ring carbon (u) E-Ring carbon (l) 1	2[a]	3	4	5[a]	6[a]
1		82.2	69.1	28.5	101.1	157.8	96.6	158.1	95.8	157.3	132.6	115.8	145.8	145.9	116.4	119.4
1a		77.5	68.1	23.8	110.0	149.3	108.6	149.7	107.5	154.8	135.9	121.6	141.9	141.9	123.5	124.3
2		78.6	67.4	29.2	100.8	157.9	96.9	158.2	96.5	157.6	132.5	115.3	145.7	145.7	115.9	120.3
2a		76.5	66.5	25.8	109.5	149.5	108.5	149.5	107.9	154.8	135.7	121.8	141.7	141.8	123.0	124.2
3		79.8	67.4	29.2	100.8	157.9	69 9	158.1	96.5	157.6	132.6	115.3	145.7	145.7	115.8	120.3
3a		76.5	66.6	25.9	109.5	149.6	108.7	149.6	107.9	154.9	135.7	121.9	141.9	141.9	123.1	124.3
4	u	104.2	66.9	29.2	102.2	152.1	98.3	156.3	96.5	156.9	131.2	115.6	144.4	145.3	116.0	119.8
	l	81.7	67.5	29.8	100.1	154.2	96.5	156.5	107.1	158.0	132.4	115.6	144.6	145.8	116.0	120.3
4a	u	105.3	66.4	26.9	97.1	148.7	109.6	150.2	107.1	153.2	135.0	122.8	141.3	142.3	122.9	125.1
	l	76.5	66.7	25.3	108.3	149.5	103.5	150.8	113.1	153.4	135.2	122.8	141.4	142.7	124.0	125.5
5	u	104.2	67.5	29.2	102.8	150.7	98.4	156.0	96.5	158.1	132.1	116.0	145.5	146.1	116.3	119.7
	l	83.9	68.5	28.7	100.3	152.1	96.5	156.2	106.5	158.1	131.1	116.0	145.8	146.6	116.3	120.3
5a	u	106.3	66.6	26.9	97.3	148.9	109.7	151.8	107.2	153.2	135.1	122.2	141.5	142.3	122.9	125.1
	l	—	68.5	28.7	100.3	152.1	98.4	156.2	106.5	158.1	131.1	116.0	145.8	146.6	116.3	120.3
6	u	104.3	66.9	29.2	102.4	152.3	98.4	154.3	96.6	156.9	132.5	115.7	145.6	146.3	116.0	119.8
	l	81.8	68.1	29.7	100.2	152.3	96.6	156.6	107.1	158.1	131.2	115.7	145.9	146.8	116.0	120.4
6a	u	105.5	66.2	27.3	97.0	148.8	109.7	150.3	107.2	153.7	135.1	122.9	141.5	142.3	122.9	125.1
	l	77.5	67.4	25.3	108.8	149.9	103.8	150.6	113.4	153.7	135.4	122.9	141.8	142.8	124.1	125.7

[a]Assignments may be interchanged, u = upper unit, l = lower unit.
—Overlapped with the solvent signal.

carbon signal of **6a** (δ77.5) with that of (+)-catechin peracetate **1a** (δ77.5), whereas the C-3 (F) signal of **6a** was observed upfield (δ67.4) compared to that of **1a** (δ68.15). The carbon signals of C-4a (D) (δ102.4) and C-4a' (δ100.2) of **6** suggested that epicatechin represents the upper unit since the analogous C-4a (A) signal of a C-4 substituted catechin unit is expected to resonate downfield at δ107 [18]. The close chemical shifts of C-3 and C-4 compared to those of proanthocyanidin A-5' (**7**) [12], mahuannins A (**8**) and B (**9**) [19] suggested that all these substances have the same relative configurations at C-2, C-3 and C-4, that is, the 2*S* 2,3-*cis* configuration. Because C-8 is observed to be more shielded than C-6 [11], the ^{13}C NMR signals of C-6 (D) and C-8 (A) (δ96.6) and C-6 (A) (δ98.4) of **6** (Table 2) corroborated the C-4/C-8 interflavan linkage structure [11]. Furthermore, the significant enhanced downfield shift of C-4 (δ27.3) in **6a** relative to the analogous signal of the parent compound **2a** (δ25.8) and the 4β analogue **5a** (δ26.9) suggested a quasi-equatorial orientation of the C-4 substituent in **6a** [18]. Confirmation of the absolute configuration at C-4 of **6a** was evident from the negative Cotton in the 200–220 nm region of its CD-spectrum consistent with a 4α configuration [20, 21]. Accordingly, this new proanthocyanidin designated as pavetannin A was unambiguously characterized as *ent*-epicatechin (4α→8,2α→*O*→7)-*ent*-catechin. Isolation of the novel metabolite **6** from *P. owariensis* represents an extension of the range of natural (2α,4α) A-type proanthocyanidins. The biosynthesis of doubly linked proanthocyanidins implying epimerization at C-2 as for **6** with (−)-epicatechin as pertative precursor, is still not clear. The co-occurrence of the (2α,4α)-representative **6** with (−)-epicatechin and *ent*-epicatechin in the same plant material, demonstrated for the first time, suggest that in addition to the C-2 epimerase enzyme postulate [7] the latter could serve as direct precursor.

EXPERIMENTAL

General. UV spectra were measured in EtOH. FABMS were recorded using *m*-nitrobenzylalcohol as liquid matrix. ^{1}H NMR (199.5 MHz) and ^{13}C NMR (50.1 MHz); for assignment of the ^{13}C NMR signals the SEFT (spin-echo Fourier transform) multipulse sequence was used, with an interval time of 7 msec (1/J), yielding positive signals for CH and Me groups, and negative signals for quaternary C atoms and CH_2 groups. Chemical shifts are reported in δ values downfield from int. TMS. CD data were obtained in MeOH. Droplet counter current chromatography was done using 300 glass columns of 40 cm length and 0.2 mm id and a flow-through microcell (250 μl hold-up vol.). CC was carried out on silica gel 60 (70–230 mesh, Merck) and Sephadex LH-20 (5–100 μ, Pharmacia). TLC was performed on either precoated aluminium sheet cellulose (0.1 mm, without fluorescent indicator, Merck) and developed with H_2O (solvent A) for the free phenolic monomers, or precoated silica gel 60 F254 plates (0.20 mm, Merck) with the following mobile phases: EtOAc–HCO_2H–HOAc–H_2O (140:2:1:59), solvent B for phenolic dimer compounds, and toluene–Me_2CO (2:1), solvent C for acetate derivatives. Spots were detected by observing their fluorescence under UV light and spraying with vanillin–H_2SO_4 or anisaldehyde–H_2SO_4 reagents. Acetylations were performed in Ac_2O-pyridine at room temp.

Isolation. Powdered stem bark of *P. owariensis* P. Beauv. 'white variety' (500 g) was percolated with Me_2CO to exhaustion. The Me_2CO extract was evapd *in vacuo* to yield a red brown powder (14 g). The residue was taken up in H_2O (500 ml) and extracted with EtOAc (3 × 1 l) to give a yellow-orange powder (2 g). This powder was subjected to CC on silica gel (stepwise elution with solvents of increasing polarity viz. $CHCl_3$, $CHCl_3$–MeOH and MeOH) yielding 15 frs of 15 ml (frs were combined according to their TLC behaviour). Frs rich in proanthocyanidins were grouped and evapd below 40° to dryness (brown amorphous powder). The residue (1.6 g) was treated with 10 ml of the upper phase of BuOH–PrOH–H_2O (2:1:3) and insol materials removed by filtration. The filtrate was applied to DCCC (ascending mode, BuOH–PrOH–H_2O, 2:1:3). The chromatographically identical frs (10 ml) were combined. Eight frs were obtained. They all showed a positive vanillin–H_2SO_4 reaction, except for fr. 1. Chromatography on Sephadex LH-20 (25–100 μ, 2 × 35 cm) with EtOH, gave compounds **4–6** from frs 3–5 and compounds **1–3** from frs 6–8. ^{1}H and ^{13}C NMR data (cf. Tables 1 and 2).

(+)-*Catechin* (**1**). Amorphous solid (5 mg); $[\alpha]_D^{20}$ +11 (Me_2CO; *c* 0.4); R_f 0.30 (solvent A); FABMS: *m/z* 291 $[M+H]^+$.

(+)-*Catechin peracetate* (**1a**). Amorphous solid (4 mg); EIMS: *m/z* 500 $[M]^{+\cdot}$, 458, 440, 398, 381, 356, 339, 314, 297, 272, 271, 223, 194, 181, 139 and 123.

(−)-*Epicatechin* (**2**). Amorphous solid (75 mg); $[\alpha]_D^{20}$ −13 (Me_2CO; *c* 0.2); R_f 0.27 (solvent A); FABMS: *m/z* 291 $[M+H]^+$.

(−)-*Epicatechin peracetate* (**2a**). Amorphous solids; EIMS: *m/z* 500 $[M]^{+\cdot}$, 458, 440, 398, 381, 356, 339, 314, 297, 272, 271, 223, 194, 181, 139 and 123.

(+)-*Epicatechin* (**3**). Amorphous solid (50 mg); $[\alpha]_D^{20}$ +10 (Me_2CO; *c* 0.2); R_f 0.24 (solvent A); FABMS: *m/z* 291 $[M+H]^+$.

(+)-*Epicatechin peracetate* (**3a**). EIMS: *m/z* 500 $[M]^+$, 458, 440, 398, 381, 356, 339, 314, 297, 272, 271, 223, 194, 181, 139 and 123.

Proanthocyanidin A-2 (**4**). Pale brown amorphous powder (360 mg); R_f 0.57 (solvent B); λ_{max}^{EtOH} 279 nm; FABMS: *m/z* 599 $[M+Na]^+$, 577 $[M+H]^+$, 425, 407 and 287.

Proanthocyanidin A-4 (**5**). Brown amorphous powder (100 mg), R_f 0.51 (solvent B); λ_{max}^{EtOH} 279 nm; FABMS: *m/z* 599 $[M+Na]^+$, *m/z* 577 $[M+H]^+$, 425, 407 and 287.

Pavetannin A (**6**). Brown amorphous powder (210 mg); R_f 0.49 (solvent B); λ_{max}^{EtOH} 279 nm; FABMS: *m/z* 599 $[M+Na]^+$, *m/z* 577 $[M+H]^+$, 425, 407 and 287; CD $[\theta]_{200}$O, $[\theta]_{205}$ −11 800, $[\theta]_{230}$O, $[\theta]_{240}$O, $[\theta]_{255}$ +590, $[\theta]_{275}$O.

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